Interview Summary	Application No.	Applicant(s)	
	09/875,823	HISERODT ET AL.	
	Examiner	Art Unit	
	Christopher H Yaen	1642	

All participants (applicant, applicant's representative, PTO personnel):

(1) *Christopher H Yaen*. (3) *Michael Schiff*.

(2) *Carol Francis*. (4)_____.

Date of Interview: *27 February 2003* .

Type: a)☒ Telephonic b)☐ Video Conference
c)☐ Personal [copy given to: 1)☐ applicant 2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☐ Yes e)☐ No.
If Yes, brief description: _____ .

Claim(s) discussed: _____ .

Identification of prior art discussed: _____ .

Agreement with respect to the claims f)☐ was reached. g)☐ was not reached. h)☒ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: *Discussed 112, 2nd 1st, and art rejections. Applcaint will provide arguments to overcome rejections. Applicant provided proposed amendments to the claims and a reference to help overcome enablement (see attached)* .

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

i)☐ It is not necessary for applicant to provide a separate record of the substance of the interview(if box is checked).

Unless the paragraph above has been checked, THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.

Christopher H Yaen
Examiner's signature, if required

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)
In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case unless both applicant and examiner agree that the examiner will record same. Where the examiner agrees to record the substance of the interview, or when it is adequately recorded on the Form or in an attachment to the Form, the examiner should check the appropriate box at the bottom of the Form which informs the applicant that the submission of a separate record of the substance of the interview as a supplement to the Form is not required.

It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

FACSIMILE TRANSMITTAL

BOZICEVIC, FIELD & FRANCIS LLP
200 Middlefield Road, Suite 200
Menlo Park, CA 94025
Telephone: (650)327-3400
Facsimile Number: (650)327-3231

Date: 2-27-03

To: Examiner Yaen

Facsimile No.: 703-~~305-3014~~ 746-7646

Phone No.:

From: Carol Francis

Our Reference: 08/901,225

Re: **Informal Communication**

Message: **PLEASE DO NOT PROCESS**
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ATTN: EXAMINER YAEN PLEASE DELIVER

1823

C ntinuation of U.S. 08/901,225 IMMEDIATELY

96-368-4 IRVN001DIV2 GET-4

Cancer Immunotherapy using Autologous Tumor Cells Combined with Allogeneic Cytokine-Secreting Cells

J.C. Hiserodt et al., University of California

FAX: 703 305 3014

Proposed Claims:

31. A method of stimulating an anti-tumor immune response or treating a neoplastic disease, comprising administering to a subject a composition comprising either a an allogeneic cell genetically altered to produce a cytokine at an elevated level, or the progeny of such a cell, wherein the cytokine is stably associated in the cell outer membrane.

32. The method of claim 31, wherein the cytokine is selected from the group consisting of IL-4, GM-CSF, IL-2, TNF-α, and M-CSF.

33. The method of claim 31, wherein the cell is a cancer cell.

34. The method of claim 31, wherein the cell is from a cancer of the same tissue type as a tumor in the subject.

35. The method of claim 3, wherein the cancer is an ovarian cancer or a brain cancer.

36. The method of claim 31, wherein the cell is allogeneic to the subject.

37. The method of claim 31, wherein the cell is histocompatibly identical to the subject.

38. The method of claim 31, wherein the composition further comprises a tumor-associated antigen, and wherein the combination of the cytokine and the tumor-associated antigen in the composition is effective in treating a neoplastic disease or eliciting an anti-tumor immunological response in the subject.

~~39. The method of claim 38, wherein the tumor-associated antigen is obtained from a cell autologous to the subject.~~

40. The method of claim 38, wherein the tumor-associated antigen is expressed by the same cells expressing the membrane-associated cytokine.

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41. The method of claim 38, wherein the composition comprises a combination of:
a) the cell expressing the membrane-associated cytokine; and
b) a tumor cell autologous to the subject;
wherein the combination is effective in treating a neoplastic disease or eliciting an anti-tumor immunological response in the subject.

42. The method of claim 41, wherein the tumor cell is a primary tumor cell dispersed from a solid tumor obtained from the subject.

43. The method of claim 41, wherein the tumor cell is a glioma, a glioblastoma, a gliosarcoma, an astrocytoma, or an ovarian cancer cell.

44. The method of claim 41, wherein the tumor cell is inactivated.

45. The method of claim 31, wherein the cell expressing the membrane-associated cytokine is inactivated.

46. The method of claim 31, wherein the cell produces a secreted cytokine in addition to the cytokine stably associated in the outer membrane.

47. The method of claim 31, wherein a majority of the cytokine produced by the cell is present on the outer membrane of the cell.

48. The method of claim 38, wherein the cytokine is selected from the group consisting of IL-4, GM-CSF, IL-2, TNF-α, and M-CSF.

49. The method of claim 31, wherein the composition comprises at least two cells, each of which has been genetically altered to produce a different cytokine at an elevated level, or is the progeny of such a cell, and wherein each cytokine is stably associated in the outer membrane of the cell.

50. A method of stimulating an anti-tumor immune response or treating a neoplastic disease, comprising administering to a subject a composition comprising a tumor associated antigen and a population of cells expressing a transmembrane cytokine at a level sufficient to stimulate an immune response to the tumor associated antigen in the subject.

51. The method of claim 31, wherein the cell is a human cell.

52. The method of claim 31, wherein the cytokine naturally occurs as a membrane cytokine.

53. The method of claim 31, wherein the cytokine is a fusion protein comprising a heterologous transmembrane region.

54. The method of claim 31, wherein the cell has been transduced with a retroviral expression vector, or is the progeny of such a cell.

55. The method of claim 31, which is a method for stimulating a primary immune response.

56. The method of claim 31, which is a method for stimulating a secondary immune response.

57. The method of claim 31, which is a method for treating a neoplastic disease.

58. The method of claim 31, further comprising providing the cytokine expressing cell that is present in the composition.

59. The method of claim 38, further comprising providing the tumor associated antigen that is present in the composition.

60. The method of claim 31, further comprising transducing a cancer cell with an expression vector encoding the membrane-associated cytokine.

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Continuation of U.S. 08/901,225

96-368-3 IRVN001DIV1 GET-3

1349

Cancer Immunotherapy using Autologous Tumor Cells Combined with Allogeneic Cytokine-Secreting Cells

J.C. Hiserodt et al., University of California

Claims:

31. A pharmaceutical composition comprising a human cell genetically altered to express a cytokine stably associated in the cell outer membrane, or the progeny of such a cell, and a pharmaceutical excipient,
formulated for administration to an allogeneic human subject;
which upon administration to a subject is effective in treating a neoplastic disease or eliciting an anti-tumor immunological response in the subject.

32. The composition of claim 31, wherein the cytokine is selected from the group consisting of IL-4, GM-CSF, IL-2, TNF-α, and M-CSF.

33. The composition of claim 31, wherein the cell is a cancer cell.

34. The composition of claim 31, wherein the cell is from a cancer of the same tissue type as a tumor in the subject.

35. The composition of claim 34, wherein the cancer is an ovarian cancer or a brain cancer.

36. The composition of claim 31, wherein the cell is allogeneic to the subject.

~~37. The composition of claim 31, wherein the cell is histocompatibly identical to the subject.~~

38. The composition of claim 31, further comprising a tumor-associated antigen, wherein the combination of the cytokine and the tumor-associated antigen in the composition is effective in treating a neoplastic disease or eliciting an anti-tumor immunological response in the subject.

39. The composition of claim 38, wherein the tumor-associated antigen is obtained from a cell autologous to the subject.

40. The composition of claim 38, wherein the tumor-associated antigen is expressed by the same cells expressing the membrane-associated cytokine.

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41. The composition of claim 38, comprising a combination of:
a) the cell expressing the membrane-associated cytokine; and
b) a tumor cell autologous to the subject;
wherein the combination is effective in treating a neoplastic disease or eliciting an anti-tumor immunological response in the subject.

42. The composition of claim 41, wherein the tumor cell is a primary tumor cell dispersed from a solid tumor obtained from the subject.

43. The composition of claim 41, wherein the tumor cell is a glioma, a glioblastoma, a gliosarcoma, an astrocytoma, or an ovarian cancer cell.

44. The composition of claim 41, wherein the tumor cell is inactivated.

45. The composition of claim 31, wherein the cell expressing the membrane-associated cytokine is inactivated.

46. The composition of claim 31, wherein the cell produces a secreted cytokine in addition to the cytokine stably associated in the outer membrane.

47. The composition of claim 31, wherein a majority of the cytokine produced by the cell is present on the outer membrane of the cell.

48. The composition of claim 38, wherein the cytokine is selected from the group consisting of IL-4, GM-CSF, IL-2, TNF-α, and M-CSF.

49. A composition comprising a tumor associated antigen and a population of cells expressing a transmembrane cytokine at a level sufficient to stimulate an immune response to the tumor associated antigen.

50. A unit dose of the composition according to claim 31, wherein the number of cells is at least about 5×10^6 but not more than about 2×10^8.

51. The composition of claim 31, wherein the cell is a human cell.

52. The composition of claim 31, wherein the cytokine naturally occurs as a membrane 5cytokine.

53. The composition of claim 31, wherein the cytokine is a fusion protein comprising a heterologous transmembrane region.

54. The composition of claim 31, wherein the cell has been transduced with a retroviral expression vector, or is the progeny of such a cell.

55. A method for producing the composition of claim 31, comprising transducing the cell with an expression vector encoding the membrane-associated cytokine.

56. The method of claim 55, wherein the expression vector is a retroviral vector.

57. The method of claim 55, wherein the cytokine is selected from the group consisting of IL-4, GM-CSF, IL-2, TNF-α, and M-CSF.

58. The method of claim 55, wherein the cytokine is expressed under control of a cytomegalovirus (CMV) promoter.

59. The method of claim 55, wherein the cell is from a cancer of the same tissue type as a tumor in the subject.

60. The method of claim 55, wherein the cell is allogeneic to the subject.

61. The method of claim 55, wherein the cell is histocompatibly identical to the subject.

62. A method for producing the composition of claim 38, comprising transducing a cell with an expression vector encoding the membrane-associated cytokine, and providing the transduced cell in combination with the tumor-associated antigen.

Tumor Cell Surface Expression of Granulocyte-Macrophage Colony-Stimulating Factor Elicits Antitumor Immunity and Protects from Tumor Challenge in the P815 Mouse Mastocytoma Tumor Model

William Soo Hoo,[1] Katherine A. Lundeen, Joshua R. Kohrumel, Nhat-Long Pham, Steven W. Brostoff, Richard M. Bartholomew, and Dennis J. Carlo

A novel membrane-bound form of GM-CSF (mbGM-CSF) was expressed on the surface of the mouse mastocytoma cell line P815 to target tumor cell-associated Ags to epidermal Langerhans cells. Transfected clones stimulated the proliferation of syngeneic bone marrow cells, indicating that mbGM-CSF is biologically active. We evaluated the in vivo effects of mbGM-CSF by comparing the growth of mbGM-CSF cells (termed 1D6.1E5) to that of wild-type P815 cells in DBA/2 mice. The growth rates of tumors initiated by P815 and 1D6.1E5 were similar until day 12, after which P815 tumors grew to large sizes while 1D6.1E5 tumors were rejected. In contrast, the growth of both tumors was unimpeded when injected into nude mice, suggesting that a T cell-dependent antitumor response was induced by 1D6.1E5 in normal mice. Lymphocytes from 1D6.1E5-vaccinated mice were able to kill ^{51}Cr-labeled P815 cells in a dose-dependent fashion that was inhibited by anti-CD8 Abs, suggesting that the antitumor response involved CD8$^+$ CTL. We then tested whether vaccination with these cells would elicit a protective antitumor response by injecting mice with either irradiated 1D6.1E5 or P815 cells and challenging them with nonirradiated P815 cells. 1D6.1E5-treated mice grew small tumors that soon disappeared in all animals. In contrast, the majority of animals receiving the irradiated wild-type tumor vaccine grew large tumors, and 50% died. These data demonstrate that mbGM-CSF expressed on the surface of tumor cells is biologically active and elicits protective antitumor immunity. *The Journal of Immunology*, 1999, 162: 7343–7349.

Preclinical tumor models using genetically modified tumor cells to secrete cytokines have been used in efforts to augment the immune response against tumor-associated Ags (1, 2). One of the more promising cytokines for the induction of potent antitumor activity is GM-CSF. This 24-kDa glycosylated cytokine has paracrine as well as autocrine effects on a number of cell types, including monocytes, dendritic cells, eosinophils, and neutrophils (3, 4). In preclinical and clinical studies, GM-CSF secreted from tumor cells has been shown to be a potent stimulator of antitumor responses (5–8). The consensus from these studies suggests that GM-CSF stimulates APC such as dendritic cells (DC)[2] to generate potent immune responses.

DC are the most potent APC in the immune system (9–11) and are able to prime naive T cells almost 30–100 times more efficiently than B cells (12, 13). Langerhans cells (LC) are immature DC that reside in the epidermis and continually sample Ag encountered in this compartment. Once LC receive the appropriate stimulus (e.g., GM-CSF, TNF-α, TGF-β, and LPS), they mature into DC (14) and migrate to lymph nodes, appearing within 24 h and peaking at 2 days after Ag uptake (15, 16), when they initiate the activation of naive T cells. These characteristics have made LC and DC the focus of intense research and attractive targets for immunotherapy. Immunotherapeutic approaches using DC include Ag pulsing of autologous DC (17, 18), transfection of DC with plasmids encoding Ags (19), and fusion of DC to tumor cells (20, 21). Taken together, these studies strongly suggest that to optimally induce an immune response using LC and DC, two requisites must be met. First, LC must be in close proximity to the appropriate Ag(s), and second, LC must receive the appropriate signals to cause maturation and migration of Ag-loaded cells to the lymph nodes to activate naive T cells.

We report here the expression and use of a novel form of GM-CSF anchored to the surface of the mouse P815 mastocytoma line through fusion with a heterologous transmembrane domain. We tested the hypothesis that tumor cells modified to express membrane-bound GM-CSF (mbGM-CSF) would effectively target tumor Ags to DC and provide an effective immune response against the unmodified parental tumor cells.

Materials and Methods

Mice

DBA/2 female mice, 8–10 wk old, and BALB/c *nu/nu* mice were purchased from Charles Rivers Laboratories (Wilmington, MA).

Antibodies

The following Abs were purchased from PharMingen (San Diego, CA). Anti-GM CSF Ab MP1-22E9 (a rat anti-mouse GM-CSF mAb), anti-CD8 Ab 53-6.7, 28.14.8 (a mouse anti-L^d Ab), SF1-1.1 (a mouse anti-K^d Ab), 34-2-12 (a mouse anti-D^d Ab), and an isotype-matched control Ab IgG2a.

Cells

P815, a mouse (H-2^d) mastocytoma derived from the DBA/2 mouse strain, was a gift from Dr. David M. Kranz (University of Illinois, Urbana, IL).

The Immune Response Corporation, Carlsbad, CA 92008

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[1] Address correspondence and reprint requests to Dr. William Soo Hoo (surname is Soo Hoo), The Immune Response Corporation, 5935 Darwin Ct., Carlsbad, CA 92008. E-mail address: youwsh@earthlink.net

[2] Abbreviations used in this paper: DC, dendritic cells; mbGM-CSF, membrane-bound GM-CSF; LC, Langerhans cells; PDGFR, platelet-derived growth factor receptor; CD40L, CD40 ligand.

0022-1767/99/$02.00

Clones 1D1 and 1D6 are mbGM-CSF-positive cells derived from P815 transfected with the vector made as described below. The clone 1D6.1E5 is a subclone derived from the 1D6 cell line by limiting dilution cloning.

Plasmid vectors

For expression of an mbGM-CSF molecule, the pHOOK-1 plasmid vector was used for cloning (Invitrogen, Carlsbad, CA). Standard DNA cloning techniques were used for construction of the vectors.

Construction of mouse mbGM-CSF

The pHOOK-1 plasmid vector originally contained the coding sequence for a single-chain Ab located between the murine κ-chain signal peptide and the platelet-derived growth factor receptor (PDGFR) transmembrane domain coding sequences. The gene encoding the single-chain Ab was removed by cutting with restriction enzymes *ApaI* and *SalI*. The resulting vector was treated with calf intestinal alkaline phosphatase (Life Technologies, Gaithersburg, MD) to remove the terminal phosphate groups.

The cDNA of murine GM-CSF was derived from PCR using BALB/c mouse mRNA from spleen cells stimulated with 4 μg/ml Con A for 2 days. The cells were lysed in RNAzol (Life Technologies), and the total RNA was extracted. Oligo(dT) primers were used to prime the synthesis of cDNA from target mRNA. The following PCR primers were used with Taq polymerase in a standard PCR reaction using a Perkin-Elmer Thermocycler (Norwalk, CT): 5′Apa mseGM-CSF, 5′-GCTAGGGCCCTAGCAC CCACCCGCTCACCCATCACT-3′; and 3′Sal mseGM-CSF, 5′-AC CGCGGTCGACTTTTTGGACTGGTTTTTTGCATTCAAAGGGG-3′. The resulting PCR fragment was purified and cloned into compatible sites in pHOOK-1 using T4 ligase (Life Technologies).

Transfection of cells

Electroporation was used for transfecting the plasmid construct into P815. Briefly, cells were grown in log phase using standard tissue culture methods in RPMI 1640 supplemented with 10% FBS and antibiotics. Cells (5×10^6) were electroporated at a voltage of 250 V in the presence of 50 μg linearized plasmid vector. Cells were then incubated with 800 μg/ml G418 (Life Technologies), and subclones were screened by FACS for the presence of GM-CSF on the surface of the cells (as described below).

Flow cytometric assays

Cells (10^6) were washed once with 2% FBS in PBS. The cells were resuspended in 50 μl of wash buffer containing 40 μg/ml rat anti-mouse GM-CSF Ab MP1-22E9 and were incubated on ice for 30 min. Cells were washed twice with wash buffer and were resuspended in wash buffer containing a fluorescein-labeled secondary Ab (goat anti-rat, mouse absorbed; Kirkegaard & Perry Laboratories, Gaithersburg, MD) and were incubated on ice 30 min. After two washes with wash buffer, the cells were resuspended in 500 μl of PBS. In some experiments cells were fixed with 4% paraformaldehyde in PBS. For flow cytometric analysis, a Becton Dickinson FACSort was used (Becton Dickinson, San Jose, CA).

Bone marrow proliferation studies

P815 or clone 1D6.1E5 cells (10^7) were incubated with 250 μg/ml mitomycin C for 30 min at 37°C. The cells were extensively washed with PBS, pH 7.2, containing 5% (v/v) FBS, resuspended in RPMI 1640 medium supplemented with 10% FBS and antibiotics, and then added to round-bottom wells at the amounts indicated. In test wells, 3×10^4 DBA/2 bone marrow cells were added, and the cultures were incubated for 2 days at 37°C in 5% CO_2 in a humidified incubator. The wells were then pulsed with 1 μCi/well [^{3}H]thymidine and harvested the next day using a Cambridge Technology PHD cell harvester (Watertown, MA).

Evaluation of live tumor growth

Wild-type P815 cells (10^6) or mbGM-CSF clones at a concentration of 20×10^6 cells/ml were injected intradermally into the hind flanks of DBA/2 mice. In other experiments, tumor cells were injected intradermally into BALB/c *nu/nu* mice. Tumors were measured three times per week over the course of the experiment starting 7–10 days after the injections. Tumor sizes are expressed as a product of the longest diameter and the shortest diameter as measured by a calibrated micrometer. Five to ten mice per group were injected.

Evaluation of irradiated tumor vaccination

Wild-type P815 cells (10^6) or mbGM-CSF clone 1D6.1E5 were irradiated with 20,000 rad in the presence of complete tissue culture medium using a JLSheperd and Associates model 109-85 Irradiator with a ^{60}Co source.

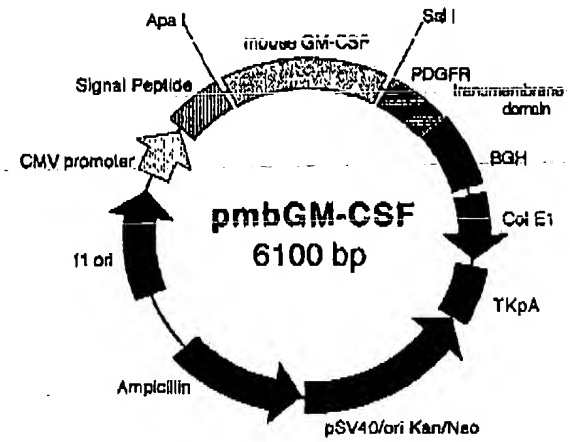

FIGURE 1. Construction of the mbGM-CSF plasmid vector. pHOOK-1 was the commercial plasmid vector into which the 372-nucleotide cDNA of GM-CSF was cloned. *ApaI* and *SalI* restriction endonuclease sites were added by PCR using primers indicated in *Materials and Methods*.

After irradiation, cells were washed with complete medium once, then extensively with PBS before injection into one flank of DBA/2 mice (10 mice/group). After 15 days the mice were boosted with the same number of cells in the same manner. Mice receiving irradiated wild-type cells were designated the control group. Five days after the boost, both groups of mice were challenged s.c. in the opposite flank with 10^6 live wild-type P815 cells. Tumors were measured as described above.

^{51}Cr release assays

Groups of five mice were vaccinated intradermally in the hind flank with 10^6 irradiated tumor cells with or without mbGM-CSF. Mice were boosted intradermally, and spleens were removed 5 days after the boost. Spleen cells were incubated with irradiated P815 cells at a ratio of 10:1 (spleen cells:tumor cells) for 5 days in complete medium. After stimulation with tumor cells, spleen cells were harvested, separated from dead cells and debris by Nycodenz density centrifugation (Accurate Chemical & Scientific Corp., Westbury, NY), then depleted of CD4$^+$ cells by Dynal magnetic bead separation (Dynal, Lake Success, NY). Target cells were prepared by adding 150 μCi of ^{51}Cr to 3×10^6 P815 cells in log phase growth for 1 h at 37°C with occasional agitation. The effector cells were then incubated at the indicated E:T cell ratios with ^{51}Cr-labeled P815 cells for 4 h in a humidified 37°C incubator. In Ab inhibition experiments, anti-CD8 Abs or a mixture of anti-class I Abs were used with the final concentrations of Abs indicated. Supernatants were then evaluated for ^{51}Cr release. The percent specific lysis was calculated as follows:

$$\% \text{ specific lysis} = \left[\frac{(\text{cpm}_{\text{experimental}} - \text{cpm}_{\text{spontaneous}})}{(\text{cpm}_{\text{maximal}} - \text{cpm}_{\text{spontaneous}})}\right] \times 100.$$

Results

Construction and expression of a membrane-anchored GM-CSF gene

The mouse GM-CSF gene consisting of 372 nucleotides was amplified from cDNA derived from Con A-stimulated mouse splenocytes, placed under control of the CMV promoter downstream of a murine Ig κ-chain signal sequence, and fused to the sequence of the PDGFR transmembrane domain (Fig. 1). We took advantage of the commercial vector, pHOOK-1, which was originally designed to express a hapten-specific single-chain Ab anchored to the plasma membrane through the PDGFR transmembrane domain (22). Cells transfected with pmbGM-CSF were selected under drug treatment, and subclones were derived from limiting dilution cloning. FACS analysis demonstrated that mbGM-CSF was expressed at levels comparable to that of the endogenous class I molecule, K^d, while isotype-matched control Abs failed to stain cells (Fig. 2). For experiments in which transfected cells were irradiated to halt

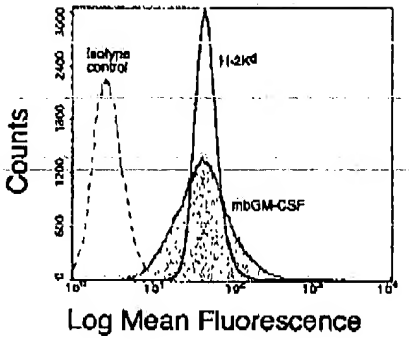

FIGURE 2. Surface expression of mbGM-CSF. P815 cells transfected with the pmbGM-CSF plasmid vector were stained with a mAb to mouse GM-CSF, anti-K^d, or isotype-matched (IgG2a) control Ab and analyzed by flow cytometry.

cell division, we observed that 20,000 rad did not alter the levels of mbGM-CSF on the surface of cells (data not shown). It is interesting to note that P815 cells express approximately 10^3 GM-CSF receptors/cell (23); however, we did not observe any significant coagulation or cell-cell clumping compared with those of wild-type cells (W.S.H. and K.A.L., unpublished observations).

mbGM-CSF cells stimulate DBA/2 bone marrow cells to proliferate in an in vitro proliferation assay

One of the important effects of GM-CSF is the stimulation of DC, resulting in their maturation marked by a rapid change from an Ag-sampling cell to APC. For efficient delivery of Ag to naive T cells, we believe it necessary for mbGM-CSF to be biologically active. Therefore, we determined whether mbGM-CSF would be able to mediate a signal to cells that are normally responsive to soluble GM-CSF. We chose to stimulate syngeneic bone marrow cells, which express the GM-CSF receptor and proliferate in the presence of soluble GM-CSF in a dose-dependent manner (24). In Fig. 3, cells expressing mbGM-CSF stimulated the proliferation of bone marrow cells, while the control wild-type P815 cells did not (Fig. 3*A*). This stimulation was specifically inhibited by the addition of an anti-GM-CSF mAb in a dose-dependent manner, whereas an isotype control Ab had no effect (Fig. 3*B*). GM-CSF also could be detected by ELISA at low levels in supernatants of membrane-bound clones (<8 pg/10^6 cells/24 h). However, these levels were orders of magnitude below that required for stimulation of bone marrow cells and approximately 4000-fold less than that reported to be biologically relevant in the elicitation of an antitumor immune response in mouse tumor models (25).

Growth rates of P815 vs 1D6.1E5 cells are unimpeded in vitro and in vivo in an immunocompromised host

We wanted to ensure that any differences we might observe in tumor growth in in vivo experiments were not due to differences in the intrinsic growth rates of the transfected cells vs wild-type cells. Therefore, the growth rates of both wild-type P815 and transfected clones were compared in vitro. Fig. 4*A* shows that the growth rates of 1D6.1E5 cells (a subclone of 1D6) were essentially the same as those of wild-type cells. Also, it made no difference whether the transfected cells were growing in the presence or the absence of the G418 selection drug. Growth rates were also determined to be equal in T cell-deficient BALB/c *nu/nu* nude mice (Fig. 4*B*). These data suggest that in the absence of a significant T cell response, cells expressing mbGM-CSF grow as solid tumors at the same rate as wild-type cells. An observation was made in all the in vivo studies with both nude and normal DBA/2 mice that there was a characteristic reduction in the mean tumor size of mice after 18–20 days. This apparent reduction was the result of the death of mice with the largest tumor burdens. We noted that the mean values of tumor size rose again, which represented the continued growth of tumors in the remaining mice.

In vivo tumor rejection of live mbGM-CSF cells in DBA/2 mice

Normally, P815 cells will grow as solid tumors when injected into a syngeneic host. It was of interest to determine whether there would be a difference between the growth of tumors initiated by transfected clones and wild-type cells. Nonirradiated P815 cells or clones bearing mbGM-CSF (designated 1D1 and 1D6) were injected intradermally into the flanks of DBA/2 mice. Initially, both mbGM-CSF cells and wild-type P815 cells grew similarly in the host. However, on day 12, tumors caused by the mbGM-CSF cells were quickly rejected, while wild-type P815 cells produced tumors that grew to significantly large sizes (Fig. 5). Indeed, for clone 1D6, 100% of animals became tumor free, while clone 1D1 showed low, but measurable, numbers of tumors. These animals were later challenged with live wild-type P815 in the opposite

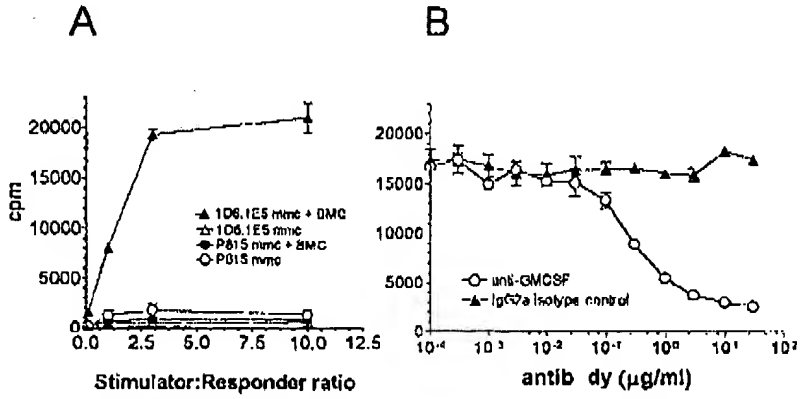

FIGURE 3. In vitro assessment of the biological activity of mbGM-CSF tumor cells. *A*, Mitomycin C-treated (mmc) stimulator cells (solid triangle, 1D6.1E5 cells; solid circle, P815 cells) were incubated in a [^{3}H]thymidine proliferation assay in the presence of 3×10^4 DBA/2 bone marrow cells (BMC) in the ratios indicated. Mitomycin C-treated stimulator cells were also cultured without BMC to control for background counts per minute (open triangle, 1D6.1E5 cells alone; open circle, P815 cells alone). *B*, BMC were stimulated by mitomycin C-treated 1D6.1E5 cells at a ratio of 3:1 (1D6.1E5: BMC) in the presence of increasing amounts of anti-GM-CSF or isotype-matched control Abs.

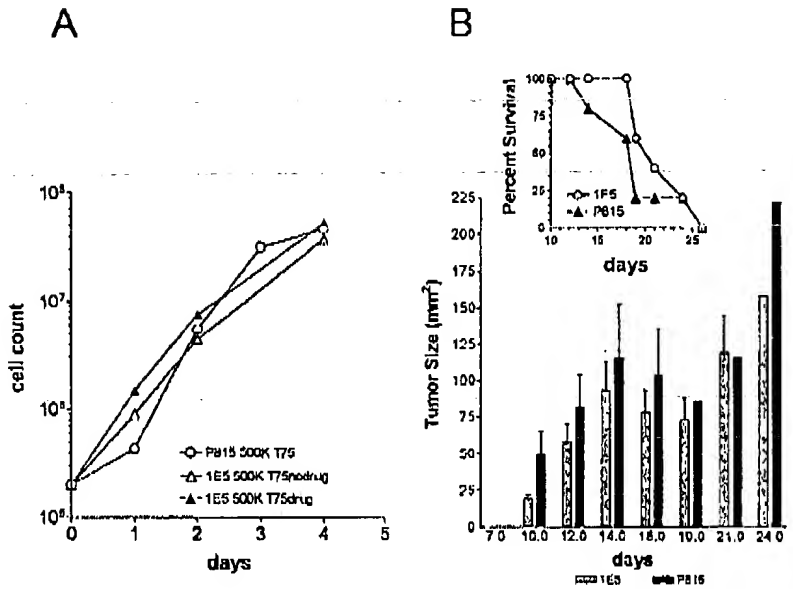

FIGURE 4. Growth comparisons of mbGM-CSF clones with wild-type P815 cells. *A*, Cells were seeded with complete medium at 5×10^5 cells/well in 75-cm² culture flasks and counted on the indicated days. *B*, 1D6.1E5 cells or P815 cells (1×10^6) were injected i.d. into BALB/c nude mice (five mice per group) and observed for tumor growth and survival.

flank and were able to reject these tumors. It was interesting that the FACS comparisons between these two clones showed that clone 1D6 expressed a significantly higher density of mbGM-CSF than did 1D1 (data not shown), which may explain the differences observed between the responses to 1D6 and 1D1 injections. For these reasons, we continued experiments with a subclone of 1D6 termed 1D6.1E5. Preliminary experiments from our laboratory using a transfected P815 cell line expressing a membrane-bound form of IL-4 (associated with a Th2 cytokine response) demonstrated that mbIL-4-expressing cells were not rejected as efficiently as cells expressing mbGM-CSF (W. Soo Hoo and J. R. Kohrumel, unpublished observations).

Vaccination with irradiated clones yields protection from a wild-type tumor challenge

Prevention of the growth of nonirradiated tumor cells may be a function of innate immunity responding to the proinflammatory effects of GM-CSF as opposed to the longer lasting effects of an adaptive immune response. To evaluate whether the mbGM-CSF-expressing cells (1D6.1E5) could elicit a protective and systemic antitumor response, irradiated cells were used to vaccinate mice before challenge with nonirradiated wild-type P815 cells. DBA/2 mice were injected intradermally with 10^6 cells in the left flank and boosted 15 days later with the same number of cells in the same flank. Five days after the last vaccination, the mice were challenged s.c. with nonirradiated wild-type tumor cells in the opposite flank. Although all mice developed palpable tumors in the first 2 wk, only animals vaccinated with mbGM-CSF cells were able to reject their tumors completely. By 30 days after the initial challenge with wild-type tumor cells, the treated group did not show any signs of tumor growth and remained tumor free throughout the remainder of the experiment. The control group, however, grew large tumors, and 50% of the mice died (Fig. 6, inset). In another experiment, mice were prevaccinated with half the dose (5×10^5 cells), and a group was added that received no vaccination before

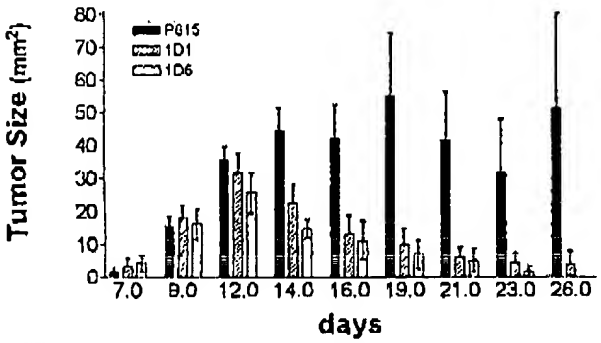

FIGURE 5. In vivo rejection of mbGM-CSF cells by syngeneic hosts. DBA/2 mice (10 mice/group) were injected with 10^6 cells intradermally in the hind flank. Tumor sizes were measured on the indicated days. Filled bars, wild-type P815; shaded bars, clone 1D1; open bars, clone 1D6.

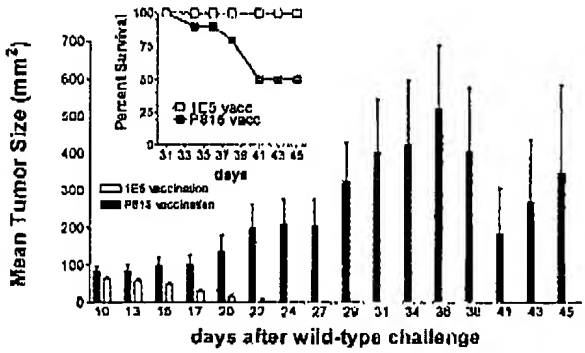

FIGURE 6. Vaccination with mbGM-CSF cells protects mice from a wild-type tumor challenge. DBA/2 mice (10 mice/group) were prevaccinated with 10^6 irradiated 1D6.1E5 or P815 cells intradermally in one hind flank. Five days after boosting in the same flank, animals were challenged s.c. with 10^6 nonirradiated P815 cells in the opposite flank. Tumors and survival were observed on the indicated days. Filled bars, P815 vaccination; open bars, 1D6.1E5 vaccination.

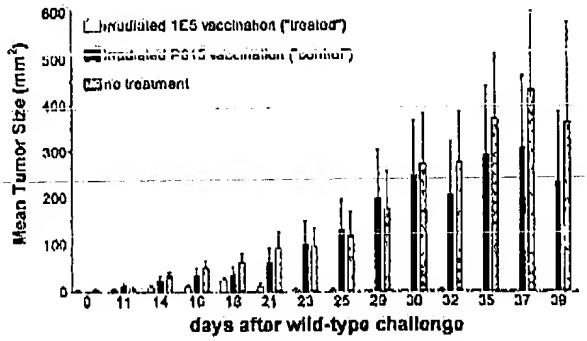

FIGURE 7. Vaccination with low doses of mbGM-CSF provides protection against a challenge of wild-type tumor cells. Irradiated 1D6.1E5 or P815 cells (5×10^5) were injected intradermally into mice (10 mice/group) and boosted. Five days later, mice were challenged s.c. with 10^6 nonirradiated P815 cells in the opposite flank, and tumors were observed on the indicated days. Open bars, 1D6.1E5 vaccination; filled bars, P815 vaccination; shaded bars, no prevaccination.

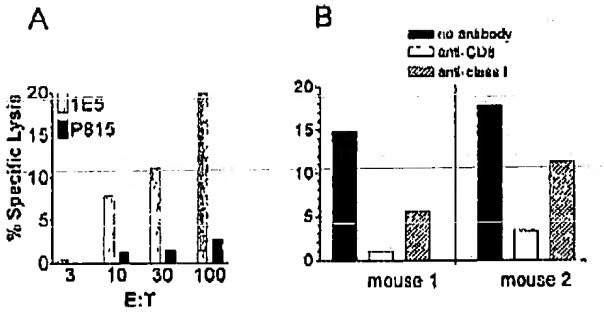

FIGURE 8. Vaccination with 1D6.1E5 cells generates wild-type tumor-specific CTL that are inhibited by anti-CD8 and anti-class I Abs. DBA/2 mice were vaccinated with 10^6 irradiated 1D6.1E5 or P815 cells and boosted on day 14. Five days after boosting, splenocytes were prepared and stimulated in vitro for 5 days in the presence of irradiated P815 cells. *A*, CTL were cultured in the presence of ^{51}Cr-labeled P815 cells in a standard 4-h chromium release assay at the indicated E:T cell ratios. *B*, Mice were primed and boosted as described above and CTL killing (E:T cell ratio, 30:1) was inhibited by specific Abs. Filled bars, no Ab added; open bars, anti-CD8 Abs (125 μg/ml); shaded bars, anti-L^d, anti-K^d, anti-D^d (each at 42 μg/ml).

challenge. Once again, the majority of mice vaccinated with the mbGM-CSF cells were able to completely reject their tumors, while no significant difference was observed between either the mice vaccinated with wild-type P815 cells or those receiving no vaccination (Fig. 7).

Antitumor activity is mediated by CTLs

In light of the results of tumor growth in nude mice and the kinetics of tumor rejection in other experiments, it seemed reasonable to assume that the antitumor activity observed in normal mice was due in large part to a proliferation of tumor-specific T cells. To test this idea, mice were immunized with 10^6 irradiated mbGM-CSF clone 1D6.1E5 or wild-type P815 cells. Five days after a boost with the same number of cells, splenocytes were isolated from all mice and given one round of stimulation by irradiated wild-type P815 cells. The question was then asked whether CTL from these preparations could kill wild-type P815 tumor cells. In Fig. 8*A*, mice that have been vaccinated with mbGM-CSF cells produce significantly more anti-P815 CTLs than mice receiving the wild-type vaccination. Anti-CD8 Abs (at a final concentration of 125 μg/ml) could inhibit the specific killing (Fig. 8*B*), indicating that the killing was $CD8^+$ CTL dependent. Furthermore, when a mixture of anti-class I Abs (L^d, K^d, D^d, each at 42 μg/ml final concentration) was used, they also inhibited the killing of P815 target cells, although to a slightly lesser degree (Fig. 8*B*). Isotype control Abs failed to inhibit CTL killing of P815 targets (data not shown). In a number of experiments, the mbGM-CSF cells consistently elicited CTL in a manner superior to P815 wild-type cells (Table I).

Discussion

To our knowledge, this is the first report of a soluble cytokine genetically engineered to be expressed in a membrane-bound form. There are examples of cytokines expressed as membrane-anchored proteins; however, these represent natural alternative splice forms (Refs. 26 and 27; reviewed in Ref. 28). Earlier work by Tao and Levy showed that GM-CSF fused to a single protein Ag was a potent immunogen and that the GM-CSF component of the fusion protein was biologically active (29). Also, the three-dimensional structure of GM-CSF described by Diedrichs et al. suggested that the molecule could be fused through its carboxyl terminus without hindering the putative receptor contact points (30). In light of this, we thought it reasonable that anchoring the GM-CSF molecule to the cell surface could be accomplished while maintaining its biological activity. Thus, we have taken advantage of the high affinity interaction between GM-CSF and its cognate receptor CD116/CDw131 ($K_d \simeq 120$ pM) (31) to target tumor Ags to epidermal LC through direct cell-cell interaction.

In this study we have demonstrated that a novel mbGM-CSF on the surface of the P815 mastocytoma cell line can generate an antitumor immune response in syngeneic DBA/2 hosts. The

Table I. *Data from three CTL assays from mice vaccinated with P815 cells with or without mbGM-CSF*[a]

	% Specific Lysis									
	3:1[b]		10:1[b]		30:1[b]		75:1[b]		100:1[b]	
Expt.	1D6.1E5	P815	1D6.1E5	P815	1D6.1E5	P815	1D6.1E5	P815	1D6.1E5	P815
I[c]	15.0	5.0	27.0	9.0	37.0	15.0	ND	ND	ND	ND
II[c]	6.5	3.0	16.0	6.7	30.0	11.0	61.0	28.0	ND	ND
III[d]	0.4	0.1	7.8	1.3	11.2	1.6	ND	ND	20.0	2.9

[a] The % specific lysis is given for each E:T ratio and for each vaccination.
[b] P815 tumor cells labeled with ^{51}Cr were used as targets in all assays.
[c] T cell cultures derived from animals vaccinated with P815 cells were stimulated in vitro with irradiated P815 cells while T cell cultures derived from animals vaccinated with 1D6.1E5 cells were stimulated in vitro with irradiated 1D6.1E5 cells.
[d] T cell cultures were stimulated in vitro with irradiated P815 cells regardless of the cells used for vaccination.

mbGM-CSF molecules are expressed at levels comparable to those of class I molecules (Fig. 2) and are able to stimulate bone marrow cells in an in vitro analysis demonstrating that the membrane-bound molecules have retained biological activity (Fig. 3). Based on our assays using soluble recombinant mouse GM-CSF, we calculate that a response yielding a stimulation index of 20 was equivalent to 2.6×10^{10} molecules of soluble GM-CSF. The number of 1D6.1E5 cells that produced the equivalent stimulation was 3×10^5 cells. Assuming that the activity/binding affinity of mbGM-CSF is the same as that of soluble GM-CSF and that each mbGM-CSF molecule on the surface has an opportunity to bind receptor, we estimate that there are 10^5 molecules of mbGM-CSF/cell. In studies using ^{125}I-radiolabeled anti-L^d mAbs, the P815 tumor line expressed approximately 6×10^5 molecules of L^d on the surface (40).

The P815 tumor cell line is known to be moderately immunogenic in the syngeneic host (32), and repeated vaccinations with wild-type P815 cells result in some antitumor immunity. For this reason, we determined that the most appropriate controls for these studies are mice vaccinated with wild-type cells. In the experiment in which nonirradiated mbGM-CSF P815 cells were injected into mice, tumors grew during the first 10–12 days. After this time, however, the tumors were quickly rejected until no palpable tumor was detected (Fig. 5), while wild-type cells grew to large sizes, resulting in 50% mortality. One explanation for this initial growth is that the priming of the immune response through the interaction of GM-CSF and DC results in a short lag period before sensitized CTL can be generated in sufficient numbers. In contrast, Nakajima et al. demonstrated that rejection of P815 cells transfected to express CD40L was immediate (i.e., no tumor growth was observed), and they showed that this rejection was due to a significant contribution of NK cells (33). When the mbGM-CSF cells were injected into BALB/c nude mice, the transfected cells grew at the same rate as wild-type cells. This is in contrast to the results seen in the Nakajima report in which CD40L-P815 cells suppressed tumor growth in nude mice, suggesting that the mechanism of antitumor immunity using mbGM-CSF is different from that used by the CD40-CD40L system. Our results are in agreement with those obtained by Sampson et al., who reported that the contribution of NK cell activity elicited by soluble GM-CSF from modified B16 melanoma cells is measurable, but relatively minor (6). This further suggests that the immunity elicited by mbGM-CSF cells is T cell dependent and that very little, if any, innate immunity is involved.

The rejection of live mbGM-CSF cells demonstrated the elicitation of an immune response directed to the modified tumor cells. This is in contrast to an initial observation in a study by Dranoff et al. in which they report that injection of live tumor cells secreting soluble GM-CSF grew progressively, inducing lethal toxicity and hepatosplenomegaly (5). However, when used as an irradiated vaccine, the GM-CSF-secreting cells induced strong antitumor immunity. From this observation one might speculate that the mbGM-CSF may be safe, since injection of live cells did not produce any lethal toxicity. Taken together with the data we have shown in athymic mice (Fig. 4*B*), we hypothesized that the rejection of live mbGM-CSF cells is mediated by a T cell-dependent systemic immune response and that this response to 1D6.1E5 cells may extend to the parental P815 cells.

The final goal of this study was to show that CTL generated using a vaccine composed of irradiated mbGM-CSF cells could elicit an immune response directed to the unmodified, parental tumor cell line both in vitro and in vivo. When used as a cell vaccine, the mbGM-CSF P815 cells were consistently superior to similar vaccinations of wild-type P815 cells in the elicitation of tumor-specific CTL (Fig. 8 and Table I) against the challenge of unmodified tumor cells. These observations are in agreement with the idea that GM-CSF is a potent cytokine adjuvant for the elicitation of antitumor responses. Since the major tumor Ags (e.g., p1A and p2Ca) of P815 are known, we plan to examine the specificity of CTL generated to the mbGM-CSF P815 cells with regard to their recognition of specific peptide Ags. The demonstration of tumor-specific CTL does not rule out the possibility that a relevant humoral immune response was also elicited. Future studies will investigate whether cells or sera from immunized animals can be adoptively transfered to convey protection from a tumor challenge or induce eradication of established tumors.

The mbGM-CSF differs from the current cytokine secretion paradigms in two distinct ways. First, contact with mbGM-CSF by GM-CSF receptors on dendritic cells requires direct physical contact with the cellular vaccine. In strategies where cytokines are secreted into the extracellular milieu, it may be tempting to speculate that DC would receive maturation signals coming from a gradient concentration of soluble GM-CSF without being close enough to take up specific Ags optimally. Also, it has been noted by others that the use of secreted, soluble cytokines requires relatively high rates of secretion for prolonged periods (~36 ng/10^6 cells/24 h) (24). Depending on the mode of transfection and the clones isolated, this may prove to be a serious limitation. The second distinction of this strategy lies in the ability to engage multiple GM-CSF receptors on a given DC. Stimulation of LC with GM-CSF causes maturation of LC in culture (34–36), resulting in the up-regulation of costimulatory molecules and increased expression of class I and class II molecules (37, 38). An additional signal is provided by the local production of TNF-α, which is responsible for the rapid migration of these cells to lymph nodes (39). We postulated that a high avidity cell-cell contact with multiple GM-CSF receptors on LC would cause the LC to experience a stronger signal transduction via the GM-CSF receptor α- and β-chains for efficient Ag uptake and rapid migration to lymph nodes. One of the aims of this system was to optimize the transduction of the signal that leads to the maturation and migration of DC. By increasing the avidity (i.e., the number of receptor-ligand interactions) between the tumor cell and the APC, we propose that the signal transduction to the DC is optimized.

The data shown here provide evidence that specific, systemic antitumor immunity can be elicited by tumor cells expressing GM-CSF on their surface. We believe that the adjuvant effect provided by mbGM-CSF is a result of the Ag-presenting DC in physical contact with the source of Ag (tumor cell), thus efficiently stimulating the antitumor response. Whether mbGM-CSF is more efficacious than secreted GM-CSF remains to be tested, and we are conducting experiments in other tumor models to directly compare the membrane-bound and secreted forms of GM-CSF.

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